

SPHINGOSINE-1-PHOSPHATE LYASE POLYPEPTIDES, POLYNUCLEOTIDES AND  
MODULATING AGENTS AND METHODS OF USE THEREFOR

5    BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to cancer detection and therapy. The invention is more particularly related to sphingosine-1-phosphate lyase polynucleotides and polypeptides, and to agents that modulate the expression and/or activity of such polypeptides. Such agents may be used, for example, to diagnose and/or treat cancers such as breast and colon cancer.

Description of the Related Art

Breast cancer is a significant health problem for women in the United States and throughout the world. Although advances have been made in detection and treatment of the disease, breast cancer remains the most common form of cancer, and the second leading cause of cancer death, in American women. Among African-American women and women between 15 and 54 years of age, breast cancer is the leading cause of cancer death. One out of every eight women in the United States will develop breast cancer, a risk which has increased 52% during 1950-1990. In 1994, it is estimated that 182,000 new cases of female breast cancer were diagnosed, and 46,000 women died from the disease.

No vaccine or other universally successful method for the prevention or treatment of breast cancer is currently available. Management of the disease currently relies on a combination of early diagnosis (through routine breast screening procedures) and aggressive treatment, which may include one or more of a variety of treatments such as surgery, radiotherapy, chemotherapy and hormone therapy. The course of treatment for a particular breast cancer is often selected based on a variety of prognostic parameters, including an analysis of

specific tumor markers. However, the use of established markers often leads to a result that is difficult to interpret.

With current therapies, tumor invasiveness and metastasis is a critical determinant in the outcome for breast cancer patients. Although the five year survival for women diagnosed with localized breast cancer is about 90%, the five year survival drops to 18% for women whose disease has metastasized. Present therapies are inadequate for inhibiting tumor invasiveness for the large population of women with this severe disease.

Colon cancer is the second most frequently diagnosed malignancy in the United States as well as the second most common cause of cancer death. The five-year survival rate for patients with colorectal cancer detected in an early localized stage is 92%; unfortunately, only 37% of colorectal cancer is diagnosed at this stage. The survival rate drops to 64% if the cancer is allowed to spread to adjacent organs or lymph nodes, and to 7% in patients with distant metastases.

The prognosis of colon cancer is directly related to the degree of penetration of the tumor through the bowel wall and the presence or absence of nodal involvement, consequently, early detection and treatment are especially important. Currently, diagnosis is aided by the use of screening assays for fecal occult blood, sigmoidoscopy, colonoscopy and double contrast barium enemas. Treatment regimens are determined by the type and stage of the cancer, and include surgery, radiation therapy and/or chemotherapy. Recurrence following surgery (the most common form of therapy) is a major problem and is often the ultimate cause of death. In spite of considerable research into therapies for the disease, colon cancer remains difficult to diagnose and treat. In spite of considerable research into therapies for these and other cancers, colon cancer remains difficult to diagnose and treat effectively. Accordingly, improvements are needed in the treatment, diagnosis and prevention of breast and colon cancer. The present invention fulfills this need and further provides other related advantages.

## BRIEF SUMMARY OF THE INVENTION

Briefly stated, the present invention provides compositions and methods for the diagnosis and therapy of cancer. Within one aspect, the present invention provides 5 isolated polynucleotides comprising a sequence selected from the group consisting of: (a) a sequence shown in SEQ ID NO:15; (b) nucleotide sequences that hybridize to a polynucleotide complementary to a sequence shown in SEQ ID NO:15 under moderately stringent conditions, wherein the nucleotide sequences encode polypeptides having sphingosine-1-phosphate lyase activity; and (c) nucleotide sequences that encode a polypeptide encoded by a sequence shown in SEQ ID NO:15.

Within a related aspect, an isolated polynucleotide is provided that encodes 10 a polypeptide shown in SEQ ID NO:16, or a variant of such a polypeptide that has sphingosine-1-phosphate lyase activity. Recombinant expression vectors comprising any of the foregoing polynucleotides, and host cells transformed or transfected with such expression vectors, are also provided.

Within further aspects, SPL polypeptides are provided. Such polypeptides 15 may be encoded by any of the foregoing polynucleotides. Alternatively, a polypeptide may comprise an amino acid sequence shown in SEQ ID NO:16, or a variant thereof, wherein the polypeptide has sphingosine-1-phosphate lyase activity.

Within a further aspect, the present invention provides isolated 20 polynucleotides comprising at least 100 nucleotides complementary to a sequence shown in SEQ ID NO:15.

Within other aspects, methods are provided for preparing a sphingosine-1- 25 phosphate lyase, comprising culturing a host cell transformed or transfected with a polynucleotide as described above under conditions promoting expression of the polynucleotide and recovering a sphingosine-1-phosphate lyase.

In further aspects, the present invention provides methods for identifying an agent that modulates sphingosine-1-phosphate lyase activity. In one such aspect, the

method comprises: (a) contacting a candidate agent with a polypeptide comprising a sequence shown in SEQ ID NO:16, or a variant of such a sequence having sphingosine-1-phosphate lyase activity, wherein the step of contacting is carried out under conditions and for a time sufficient to allow the candidate agent to interact with the polypeptide; and (b) 5 subsequently measuring the ability of the polypeptide to degrade sphingosine-1-phosphate or a derivative thereof, relative to an ability in the absence of candidate agent. The step of contacting may be performed by incubating a cell expressing the polypeptide with the candidate modulator, and the step of measuring the ability to degrade sphingosine-1-phosphate may be performed using an *in vitro* assay and a cellular extract.

10 The present invention further provides pharmaceutical compositions comprising an agent that modulates sphingosine-1-phosphate lyase activity in combination with a pharmaceutically acceptable carrier. Such agents preferably increase sphingosine-1-phosphate lyase activity. Such inhibition may be achieved by increasing expression of an endogenous SPL gene, or by increasing the ability of an endogenous SPL to degrade sphingosine-1-phosphate. Within certain preferred embodiments, a modulating agent 15 comprises a polynucleotide or an antibody or an antigen-binding fragment thereof.

20 Within still further aspects, the present invention provides methods for modulating sphingosine-1-phosphate activity, comprising contacting a sphingosine-1-phosphate lyase with an effective amount of an agent that modulates sphingosine-1-phosphate lyase activity, wherein the step of contacting is performed under conditions and for a time sufficient to allow the agent and the sphingosine-1-phosphate lyase to interact. To modulate sphingosine-1-phosphate lyase activity in a cell, a cell expressing sphingosine-1-phosphate may be contacted with such an agent.

25 Within related aspects, the present invention provides methods for inhibiting the growth of a cancer cell, comprising contacting a cancer cell with an agent that increases sphingosine-1-phosphate lyase activity. In a preferred embodiment, the cancer cell is a breast cancer cell.

The present invention also provides methods for inhibiting the development and/or metastasis of a cancer in a mammal, comprising administering to a mammal an agent that increases sphingosine-1-phosphate lyase activity. Within certain embodiments, an agent may comprise, or be linked to, a targeting component, such as an anti-tumor antibody or a component that binds to an estrogen receptor.

Within other aspects, methods for diagnosing cancer in a mammal are provided, comprising detecting an alteration in an endogenous sphingosine-1-phosphate lyase gene in a sample obtained from a mammal, and therefrom diagnosing a cancer in the mammal. In certain embodiments the cancer is breast or colon cancer and the sample is a breast tumor biopsy.

In related aspects, the present invention provides methods for evaluating a cancer prognosis, comprising determining the presence or absence of an alteration in an endogenous sphingosine-1-phosphate lyase gene in a sample obtained from a mammal afflicted with cancer, and therefrom determining a prognosis.

The present invention further provides isolated antibodies that bind to a polypeptide having a sequence shown in SEQ ID NO:16. Such antibodies may be polyclonal or monoclonal, and may increase the ability of a polypeptide having a sequence shown in SEQ ID NO:16 degrade sphingosine-1-phosphate.

In still further aspects, the present invention provides methods for detecting sphingosine-1-phosphate lyase in a sample, comprising: (a) contacting a sample with an antibody as described above under conditions and for a time sufficient to allow the antibody to bind to sphingosine-1-phosphate lyase; and (b) detecting in the sample the presence of sphingosine-1-phosphate lyase bound to the antibody.

Kits for use in the above methods are also provided. A kit for detecting sphingosine-1-phosphate lyase in a sample comprises an antibody as described above and a buffer or detection reagent. A kit for detecting an alteration in a sphingosine-1-phosphate gene in a sample comprises a polynucleotide and a detection reagent.

The present invention further provides for a homozygous null mutant *Drosophila melanogaster* fly line the genome of which comprises a P-element transposon insertion in the coding region of the sphingosine phosphate lyase (SPL) gene wherein said gene encodes the sequence set forth in SEQ ID NO:16, and wherein said fly line has a 5 flightless phenotype. In a related embodiment, the homozygous mutant flies demonstrate abnormal developmental patterning of thoracic muscles of the T2 segment.

The present invention also provides methods for testing an agent capable of inhibiting the development and/or metastasis of a cancer in a mammal, comprising contacting SPL mutant *Drosophila* progeny with growth medium comprising a test agent suspected of inhibiting mammalian sphingosine kinase, and detecting the restoration of flight ability in the progeny. In a related embodiment, the homozygous mutant flies used in this method demonstrate abnormal developmental patterning of thoracic muscles of the T2 segment.

The present invention further provides for methods for determining the presence 15 of a cancer in a patient, comprising the steps of: (a) obtaining a biological sample from the patient; (b) contacting the biological sample with at least one oligonucleotide that is at least partially complementary to the sequence set forth in SEQ ID NO:7; (c) detecting in the sample an amount of said oligonucleotide that hybridizes to the polynucleotide; and comparing the amount 20 of oligonucleotide that hybridizes to the polynucleotide to a predetermined cut-off value, and therefrom determining the presence of the cancer in the patient.

#### BRIEF DESCRIPTION OF THE SEQUENCE IDENTIFIERS

SEQ ID NO:1 is the determined cDNA sequence of *S. cerevisiae* SPL  
25 SEQ ID NO:2 is the amino acid sequence of *S. cerevisiae* SPL encoded by the polynucleotide sequence set forth in SEQ ID NO:1  
SEQ ID NO:3 is the determined cDNA sequence of *C. elegans* SPL

SEQ ID NO:4 is the amino acid sequence of *C. elegans* SPL encoded by the polynucleotide sequence set forth in SEQ ID NO:3

SEQ ID NO:5 is the determined cDNA sequence of the mouse SPL

SEQ ID NO:6 is the amino acid sequence of mouse SPL encoded by the  
5 polynucleotide sequence set forth in SEQ ID NO:5

SEQ ID NO:7 is the determined cDNA sequence of the full-length human SPL

SEQ ID NO:8 is the amino acid sequence of human SPL encoded by the polynucleotide sequence set forth in SEQ ID NO:7

SEQ ID NO:9 is the determined cDNA sequence of a human SPL with a deletion

10 SEQ ID NO:10 is the amino acid sequence of a human SPL with a deletion,  
encoded by the polynucleotide sequence set forth in SEQ ID NO:9

SEQ ID NO:11 is the amino acid sequence of *C. elegans* SPL encoded by the polynucleotide sequence set forth in SEQ ID NO:12

SEQ ID NO:12 is the determined cDNA sequence of a *C. elegans* SPL

15 SEQ ID NO:13 is a PCR primer

SEQ ID NO:14 is a PCR primer

SEQ ID NO:15 is the determined cDNA sequence encoding the *Drosophila melanogaster* SPL

20 SEQ ID NO:16 is the amino acid sequence of the *Drosophila melanogaster* SPL,  
encoded by the cDNA sequence set forth in SEQ ID NO:15

SEQ ID NO:17 is the determined cDNA sequence of a human SPL as set forth in  
Genbank Accession No: AF144638.

25 SEQ ID NO:18 is the amino acid sequence of a human SPL encoded by the  
polynucleotide sequence provided in SEQ ID NO:17.

SEQ ID NO:19 is the amino acid sequence of a first *Drosophila melanogaster* SK  
protein.

SEQ ID NO:20 is the amino acid sequence of a second *Drosophila melanogaster*  
SK protein.

SEQ ID NO:21 is the amino acid sequence of a human SK protein.

#### DETAILED DESCRIPTION OF THE INVENTION

As noted above, the present invention is generally directed to compositions and methods for the diagnosis and therapy of cancers such as breast cancer. The invention is more particularly related to sphingosine-1-phosphate lyase (SPL) polypeptides, which have the ability to cleave sphingosine-1-phosphate into inactive metabolites, and to polynucleotides encoding such polypeptides. Sphingosine-1-phosphate (S-1-P) is an endogenous sphingolipid metabolite present in most mammalian cells and in serum. Like other sphingolipid metabolites such as ceramide and sphingosine, S-1-P participates in specific signal transduction pathways. The results of S-1-P signaling are diverse and dependent upon the cell type being examined. However, many of the effects of S-1-P signaling, which include promotion of cellular proliferation, enhancement of migration, inhibition of apoptosis and stimulation of angiogenesis, influence the transformation, growth, drug resistance, vascularity and metastatic capacity of cancer cells. The gene encoding the enzyme responsible for S-1-P synthesis is sphingosine kinase, SK, and S-1-P degradation is sphingosine phosphate lyase, SPL and S-1-P phosphatase, S-1-PP. Several observations support the notion that SPL may be a cancer related gene. First, altered expression of SPL in human tumors compared to corresponding normal tissue from the same patient has been shown. Second, human SPL maps to 10q21, a chromosomal region frequently deleted in a variety of human cancers. Taken together, these observations raise the possibility that SPL may be potentially effective targets for pharmacological intervention in the treatment of cancer.

Agents that decrease the expression or activity of endogenous SPL polypeptides are encompassed by the present invention. Such modulating agents may be identified using methods described herein and used, for example, in cancer therapy. It has also been found, within the context of the present invention, that the detection of alterations in an endogenous SPL sequence can be used to diagnose cancer, and to assess the prognosis for recovery. The present invention further provides such diagnostic methods and kits.

As used herein, the term "polypeptide" encompasses amino acid chains of any length, including full length endogenous (*i.e.*, native) SPL proteins and variants of endogenous sequences. "Variants" are polypeptides that differ in sequence from a native SPL only in substitutions, deletions and/or other modifications, such that the variant retains SPL activity, 5 which may be determined using a representative method described herein. SPL polypeptide variants generally encompassed by the present invention will typically exhibit at least about 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or more identity along its length, to an SPL polypeptide sequence set forth herein. Within an SPL polypeptide variant, amino acid substitutions are preferably made at no more than 50% of the amino acid residues in the native polypeptide, and more preferably at no more than 25% of the amino acid residues. Such substitutions are preferably conservative. A conservative substitution is one in which an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydrophobic nature of the polypeptide to be substantially unchanged. In general, the following amino acids 10 represent conservative changes: (1) ala, pro, gly, glu, asp, gln, asn, ser, thr; (2) cys, ser, tyr, thr; (3) val, ile, leu, met, ala, phe; (4) lys, arg, his; and (5) phe, tyr, trp, his. Substitutions, deletions and/or amino acid additions may be made at any location(s) in the polypeptide, provided that the 15 modification does not diminish the SPL activity of the variant. Thus, a variant may comprise only a portion of a native SPL sequence. In addition, or alternatively, variants may contain additional amino acid sequences (such as, for example, linkers, tags and/or ligands), preferably at 20 the amino and/or carboxy termini. Such sequences may be used, for example, to facilitate purification, detection or cellular uptake of the polypeptide.

When comparing polypeptide sequences, two sequences are said to be "identical" if the sequence of amino acids in the two sequences is the same when aligned for maximum 25 correspondence, as described below. Comparisons between two sequences are typically performed by comparing the sequences over a comparison window to identify and compare local regions of sequence similarity. A "comparison window" as used herein, refers to a segment of at least about 20 contiguous positions, usually 30 to about 75, 40 to about 50, in which a sequence

may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned.

Optimal alignment of sequences for comparison may be conducted using the Megalign program in the Lasergene suite of bioinformatics software (DNASTAR, Inc., Madison, WI), using default parameters. This program embodies several alignment schemes described in the following references: Dayhoff, M.O. (1978) A model of evolutionary change in proteins – Matrices for detecting distant relationships. In Dayhoff, M.O. (ed.) *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, Washington DC Vol. 5, Suppl. 3, pp. 345-358; Hein J. (1990) Unified Approach to Alignment and Phylogenies pp. 626-645 *Methods in Enzymology* vol. 183, Academic Press, Inc., San Diego, CA; Higgins, D.G. and Sharp, P.M. (1989) *CABIOS* 5:151-153; Myers, E.W. and Muller W. (1988) *CABIOS* 4:11-17; Robinson, E.D. (1971) *Comb. Theor* 11:105; Saitou, N. Nei, M. (1987) *Mol. Biol. Evol.* 4:406-425; Sneath, P.H.A. and Sokal, R.R. (1973) *Numerical Taxonomy – the Principles and Practice of Numerical Taxonomy*, Freeman Press, San Francisco, CA; Wilbur, W.J. and Lipman, D.J. (1983) *Proc. Natl. Acad. Sci. USA* 80:726-730.

Alternatively, optimal alignment of sequences for comparison may be conducted by the local identity algorithm of Smith and Waterman (1981) *Add. APL. Math* 2:482, by the identity alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443, by the search for similarity methods of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. USA* 85: 2444, by computerized implementations of these algorithms (GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, WI), or by inspection.

One preferred example of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al. (1977) *Nucl. Acids Res.* 25:3389-3402 and Altschul et al. (1990) *J. Mol. Biol.* 215:403-410, respectively. BLAST and BLAST 2.0 can be used, for example with the parameters described herein, to determine percent sequence identity for the polynucleotides and polypeptides of the invention. Software for performing BLAST analyses is publicly available

through the National Center for Biotechnology Information. For amino acid sequences, a scoring matrix can be used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation 5 of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment.

The SPL activity of an SPL polypeptide or variant thereof may generally be assessed using an *in vitro* assay that detects the degradation of labeled substrate (*i.e.*, sphingosine-1-phosphate, or a derivative thereof). Within such assays, pyridoxal 5'-phosphate is a requirement for SPL activity. In addition, the reaction generally proceeds optimally at pH 7.4-10 7.6 and requires chelators due to sensitivity toward heavy metal ions. The substrate should be a D-*erythro* isomer, but in derivatives of sphingosine-1-phosphate the type and chain length of sphingoid base may vary. In general, an assay as described by Van Veldhoven and Mannaerts, *J. 15 Biol. Chem.* 266:12502-07, 1991 may be employed. Briefly, a solution (*e.g.*, a cellular extract) containing the polypeptide may be incubated with 40  $\mu$ M substrate at 37°C for 1 hour in the presence of, for example, 50 mM sucrose, 100 mM K-phosphate buffer pH 7.4, 25 mM NaF, 0.1% (w/v) Triton X-100, 0.5 mM EDTA, 2 mM DTT, 0.25 mM pyridoxal phosphate. Reactions may then be terminated and analyzed by thin-layer chromatography to detect the formation of 20 labeled fatty aldehydes and further metabolites. In general, a polypeptide has SPL activity if, within such an assay: (1) the presence of 2 - 50  $\mu$ g polypeptide (or 0.1 - 10 mg/mL) results in a statistically significant increase in the level of substrate degradation, preferably a two-fold increase, relative to the level observed in the absence of polypeptide; and (2) the increase in the level of substrate degradation is pyridoxal 5'-phosphate dependent.

25 Within certain embodiments, an *in vitro* assay for SPL activity may be performed using cellular extracts prepared from cells that express the polypeptide of interest. Preferably, in the absence of a gene encoding an SPL polypeptide, such cells do not produce a significant amount of endogenous SPL (*i.e.*, a cellular extract should not contain a detectable increase in the

level of SPL, as compared to buffer alone without extract). It has been found, within the context of the present invention, that yeast cells containing deletion of the SPL gene (*BST1*) are suitable for use in evaluating the SPL activity of a polypeptide. *bst1* $\Delta$  cells can be generated from *S. cerevisiae* using standard techniques, such as PCR, as described herein. A polypeptide to be tested for SPL activity may then be expressed in *bst1* $\Delta$  cells, and the level of SPL activity in an extract containing the polypeptide may be compared to that of an extract prepared from cells that do not express the polypeptide. For such a test, a polypeptide is preferably expressed on a high-copy yeast vector (such as pYES2, which is available from Invitrogen) yielding more than 20 copies of the gene per cell. In general, a polypeptide has SPL activity if, when expressed using such a vector in a *bst1* $\Delta$  cell, a cellular extract results in a two-fold increase in substrate degradation over the level observed for an extract prepared from cells not expressing the polypeptide.

A further test for SPL activity may be based upon functional complementation in the *bst1* $\Delta$  strain. It has been found, within the context of the present invention, that *bst1* $\Delta$  cells are highly sensitive to D-*erythro*-sphingosine. In particular, concentrations as low as 10  $\mu$ M sphingosine completely inhibit the growth of *bst1* $\Delta$  cells. Such a level of sphingosine has no effect on the growth of wildtype cells. A polypeptide having SPL activity as provided above significantly diminishes (*i.e.*, by at least two fold) the sphingosine sensitivity when expressed on a high-copy yeast vector yielding more than 20 copies of the gene per cell.

In general, SPL polypeptides, and polynucleotides encoding such polypeptides, may be prepared using any of a variety of techniques that are well known in the art. For example, a DNA sequence encoding native SPL may be prepared by amplification from a suitable cDNA or genomic library using, for example, polymerase chain reaction (PCR) or hybridization techniques. Libraries may generally be prepared and screened using methods well known to those of ordinary skill in the art, such as those described in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 1989. cDNA libraries may be prepared from any of a variety of sources known to contain enzymes having SPL activity. SPL activity is ubiquitous with regard to species and mammalian

tissues, with the exception of platelets, in which SPL activity is notably absent. In rat tissues, the highest levels of activity have been demonstrated in intestinal mucosa, liver and Harderian gland, with low activity in skeletal muscle and heart. Activity has also been demonstrated in a number of human (hepatoma cell line HB 8065, cervical carcinoma HeLa), mouse (hepatoma line BW1, 5 mouse embryo 3T3-L1, Swiss 3T3 cells) and other cell lines, as well as in human cultured fibroblasts. Preferred cDNA libraries may be prepared from human liver, intestine or brain tissues or cells. Other libraries that may be employed will be apparent to those of ordinary skill in the art. Primers for use in amplification may be readily designed based on the sequence of a native SPL polypeptide or polynucleotide, as provided herein.

10 Alternatively, an endogenous SPL gene may be identified using a screen for cDNAs that complement the *BST1* deletion in yeast. A cDNA expression library may be generated using a regulatable yeast expression vector (e.g., pYES, which is available from Invitrogen, Inc.) and standard techniques. A yeast *bst1* $\Delta$  strain may then be transformed with the cDNA library, and endogenous cDNAs having the ability to functionally complement the yeast 15 lyase defect (i.e., restore the ability to grow in the presence of D-*erythro*-sphingosine) may be isolated.

20 An endogenous SPL gene may also be identified based on cross-reactivity of the protein product with anti-SPL antibodies, which may be prepared as described herein. Such screens may generally be performed using standard techniques (see Huynh et al., "Construction and Screening cDNA Libraries in  $\lambda$ gt11," in D.M. Glover, ed., *DNA Cloning: A Practical Approach*, 1:49-78, 1984 (IRL Press, Oxford)).

25 Polynucleotides encompassed by the present invention include DNA and RNA molecules that comprise an endogenous SPL gene sequence. Such polynucleotides include those that comprise a sequence recited in any one of SEQ ID NOs:1-16. Also encompassed are other polynucleotides that encode an SPL amino acid sequence encoded by such polynucleotides, as well as polynucleotides that encode variants of a native SPL sequence that retain SPL activity. Polynucleotides that are substantially homologous to a sequence complementary to an endogenous SPL gene are also within the scope of the present invention. "Substantial

homology," as used herein refers to polynucleotides that are capable of hybridizing under moderately stringent conditions to a polynucleotide complementary to an SPL polynucleotide sequence provided herein, provided that the encoded SPL polypeptide variant retains SPL activity. Suitable moderately stringent conditions include prewashing in a solution of 5X SSC, 5 0.5% SDS, 1.0 mM EDTA (pH 8.0); hybridizing at 50-65°C, 5X SSC, overnight; followed by washing twice at 65°C for 20 minutes with each of 2X, 0.5X and 0.2X SSC containing 0.1% SDS. Nucleotide sequences that, because of code degeneracy, encode a polypeptide encoded by any of the above sequences are also encompassed by the present invention.

10 Polypeptides of the present invention may be prepared by expression of recombinant DNA encoding the polypeptide in cultured host cells. Preferably, the host cells are bacteria, yeast, insect or mammalian cells, and more preferably the host cells are *S. cerevisiae bst1Δ* cells. The recombinant DNA may be cloned into any expression vector suitable for use within the host cell and transfected into the host cell using techniques well known to those of ordinary skill in the art. A suitable expression vector contains a promoter sequence that is active 15 in the host cell. A tissue-specific or conditionally active promoter may also be used. Preferred promoters express the polypeptide at high levels.

20 Optionally, the construct may contain an enhancer, a transcription terminator, a poly(A) signal sequence, a bacterial or mammalian origin of replication and/or a selectable marker, all of which are well known in the art. Enhancer sequences may be included as part of the promoter region or separately. Transcription terminators are sequences that stop RNA 25 polymerase-mediated transcription. The poly(A) signal may be contained within the termination sequence or incorporated separately. A selectable marker includes any gene that confers a phenotype on the host cell that allows transformed cells to be identified. Such markers may confer a growth advantage under specified conditions. Suitable selectable markers for bacteria are well known and include resistance genes for ampicillin, kanamycin and tetracycline. Suitable selectable markers for mammalian cells include hygromycin, neomycin, genes that complement a deficiency in the host (e.g., thymidine kinase and TK<sup>-</sup>cells) and others well known in the art. For

yeast cells, one suitable selectable marker is URA3, which confers the ability to grow on medium without uracil.

DNA sequences expressed in this manner may encode a native SPL polypeptide (e.g., human), or may encode portions or other variants of native SPL polypeptide. DNA molecules encoding variants of a native SPL may generally be prepared using standard mutagenesis techniques, such as oligonucleotide-directed site-specific mutagenesis, and sections of the DNA sequence may be removed to permit preparation of truncated polypeptides.

To generate cells that express a polynucleotide encoding an SPL polypeptide, cells may be transfected, transformed or transduced using any of a variety of techniques known in the art. Any number of transfection, transformation, and transduction protocols known to those in the art may be used, for example those outlined in Current Protocols in Molecular Biology, John Wiley & Sons, New York, N.Y., or in numerous kits available commercially (e.g., Invitrogen Life Technologies, Carlsbad, CA). Such techniques may result in stable transformants or may be transient. One suitable transfection technique is electroporation, which may be performed on a variety of cell types, including mammalian cells, yeast cells and bacteria, using commercially available equipment. Optimal conditions for electroporation (including voltage, resistance and pulse length) are experimentally determined for the particular host cell type, and general guidelines for optimizing electroporation may be obtained from manufacturers. Other suitable methods for transfection will depend upon the type of cell used (e.g., the lithium acetate method for yeast), and will be apparent to those of ordinary skill in the art. Following transfection, cells may be maintained in conditions that promote expression of the polynucleotide within the cell. Appropriate conditions depend upon the expression system and cell type, and will be apparent to those skilled in the art.

SPL polypeptides may be expressed in transfected cells by culturing the cell under conditions promoting expression of the transfected polynucleotide. Appropriate conditions will depend on the specific host cell and expression vector employed, and will be readily apparent to those of ordinary skill in the art. For commercially available expression vectors, the polypeptide may generally be expressed according to the manufacturer's instructions. For certain purposes,

10  
15  
20  
25

expressed polypeptides of this invention may be isolated in substantially pure form. Preferably, the polypeptides are isolated to a purity of at least 80% by weight, more preferably to a purity of at least 95% by weight, and most preferably to a purity of at least 99% by weight. In general, such purification may be achieved using, for example, the standard techniques of ammonium sulfate fractionation, SDS-PAGE electrophoresis, and/or affinity chromatography.

The present invention further provides antibodies that bind to an SPL polypeptide. Antibodies may function as modulating agents (as discussed further below) to inhibit or block SPL activity *in vivo*. Alternatively, or in addition, antibodies may be used within screens for endogenous SPL polypeptides or modulating agents, for purification of SPL polypeptides, for assaying the level of SPL within a sample and/or for studies of SPL expression. Such antibodies may be polyclonal or monoclonal, and are generally specific for one or more SPL polypeptides and/or one or more variants thereof. Within certain preferred embodiments, antibodies are polyclonal.

Antibodies may be prepared by any of a variety of techniques known to those of ordinary skill in the art (see, e.g., Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988). In one such technique, an immunogen comprising an SPL polypeptide or antigenic portion thereof is initially injected into a suitable animal (e.g., mice, rats, rabbits, sheep and goats), preferably according to a predetermined schedule incorporating one or more booster immunizations. The use of rabbits is preferred. To increase immunogenicity, an immunogen may be linked to, for example, glutaraldehyde or keyhole limpet hemocyanin (KLH). Following injection, the animals are bled periodically to obtain post-immune serum containing polyclonal anti-SPL antibodies. Polyclonal antibodies may then be purified from such antisera by, for example, affinity chromatography using an SPL polypeptide or antigenic portion thereof coupled to a suitable solid support. Such polyclonal antibodies may be used directly for screening purposes and for Western blots.

More specifically, an adult rabbit (e.g., NZW) may be immunized with 10 µg purified (e.g., using a nickel-column) SPL polypeptide emulsified in complete Freund's adjuvant (1:1 v/v) in a volume of 1mL. Immunization may be achieved via injection in at least six

different subcutaneous sites. For subsequent immunizations, 5  $\mu$ g of an SPL polypeptide may be emulsified in complete Freund's adjuvant and injected in the same manner. Immunizations may continue until a suitable serum antibody titer is achieved (typically a total of about three immunizations). The rabbit may be bled immediately before immunization to obtain pre-immune serum, and then 7-10 days following each immunization.

For certain embodiments, monoclonal antibodies may be desired. Monoclonal antibodies may be prepared, for example, using the technique of Kohler and Milstein, *Eur. J. Immunol.* 6:511-519, 1976, and improvements thereto. Briefly, these methods involve the preparation of immortal cell lines capable of producing antibodies having the desired specificity (i.e., reactivity with the polypeptide of interest). Such cell lines may be produced, for example, from spleen cells obtained from an animal immunized as described above. The spleen cells are then immortalized by, for example, fusion with a myeloma cell fusion partner, preferably one that is syngeneic with the immunized animal. For example, the spleen cells and myeloma cells may be combined with a nonionic detergent for a few minutes and then plated at low density on a selective medium that supports the growth of hybrid cells, but not myeloma cells. A preferred selection technique uses HAT (hypoxanthine, aminopterin, thymidine) selection. After a sufficient time, usually about 1 to 2 weeks, colonies of hybrids are observed. Single colonies are selected and tested for binding activity against the polypeptide. Hybridomas having high reactivity and specificity are preferred.

Monoclonal antibodies may be isolated from the supernatants of growing hybridoma colonies. In addition, various techniques may be employed to enhance the yield, such as injection of the hybridoma cell line into the peritoneal cavity of a suitable vertebrate host, such as a mouse. Monoclonal antibodies may then be harvested from the ascites fluid or the blood. Contaminants may be removed from the antibodies by conventional techniques, such as chromatography, gel filtration, precipitation, and extraction.

As noted above, the present invention provides agents that modulate, preferably inhibit, the expression (transcription or translation), stability and/or activity of an SPL polypeptide. To identify such a modulating agent, any of a variety of screens may be performed.

Candidate modulating agents may be obtained using well known techniques from a variety of sources, such as plants, fungi or libraries of chemicals, small molecules or random peptides. Antibodies that bind to an SPL polypeptide, and anti-sense polynucleotides that hybridize to a polynucleotides that encodes an SPL, may be candidate modulating agents. Preferably, a modulating agent has a minimum of side effects and is non-toxic. For some applications, agents that can penetrate cells are preferred.

Screens for modulating agents that decrease SPL expression or stability may be readily performed using well known techniques that detect the level of SPL protein or mRNA. Suitable assays include RNase protection assays, *in situ* hybridization, ELISAs, Northern blots and Western blots. Such assays may generally be performed using standard methods (see Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 1989). For example, to detect mRNA encoding SPL, a nucleic acid probe complementary to all or a portion of the SPL gene sequence may be employed in a Northern blot analysis of mRNA prepared from suitable cells. Alternatively, real-time PCR can also be used to detect levels of mRNA encoding SPL (see Gibson et al., *Genome Research* 6:995-1001, 1996; Heid et al., *Genome Research* 6:986-994, 1996). The first-strand cDNA to be used in the quantitative real-time PCR is synthesized from 20 $\mu$ g of total RNA that is first treated with DNase I (e.g., Amplification Grade, Gibco BRL Life Technology, Gaithersburg, MD), using Superscript Reverse Transcriptase (RT) (e.g., Gibco BRL Life Technology, Gaithersburg, MD). Real-time PCR is performed, for example, with a GeneAmp<sup>TM</sup> 5700 sequence detection system (PE Biosystems, Foster City, CA). The 5700 system uses SYBR<sup>TM</sup> green, a fluorescent dye that only intercalates into double stranded DNA, and a set of gene-specific forward and reverse primers. The increase in fluorescence is monitored during the whole amplification process. The optimal concentration of primers is determined using a checkerboard. The PCR reaction is performed in 25 $\mu$ l volumes that include 2.5 $\mu$ l of SYBR green buffer, 2 $\mu$ l of cDNA template and 2.5 $\mu$ l each of the forward and reverse primers for the SPL gene, or other gene of interest. The cDNAs used for RT reactions are diluted approximately 1:10 for each gene of interest and 1:100 for the  $\beta$ -actin control. In order to quantitate the amount of specific cDNA (and hence initial

mRNA) in the sample, a standard curve is generated for each run using the plasmid DNA containing the gene of interest. Standard curves are generated using the Ct values determined in the real-time PCR which are related to the initial cDNA concentration used in the assay. Standard dilution ranging from 20-2x10<sup>6</sup> copies of the SPL gene or other gene of interest are used for this purpose. In addition, a standard curve is generated for β-actin ranging from 200fg-2000fg. This enables standardization of the initial RNA content of a sample to the amount of β-actin for comparison purposes. The mean copy number for each sample tested is normalized to a constant amount of β-actin, allowing the evaluation of the observed expression levels of SPL or other gene of interest.

To detect SPL protein, a reagent that binds to the protein (typically an antibody, as described herein) may be employed within an ELISA or Western assay. Following binding, a reporter group suitable for direct or indirect detection of the reagent is employed (*i.e.*, the reporter group may be covalently bound to the reagent or may be bound to a second molecule, such as Protein A, Protein G, immunoglobulin or lectin, which is itself capable of binding to the reagent). Suitable reporter groups include, but are not limited to, enzymes (*e.g.*, horseradish peroxidase), substrates, cofactors, inhibitors, dyes, radionuclides, luminescent groups, fluorescent groups and biotin. Such reporter groups may be used to directly or indirectly detect binding of the reagent to a sample component using standard methods known to those of ordinary skill in the art.

To use such assays for identifying a modulating agent, the level of SPL protein or mRNA may be evaluated in cells treated with one or more candidate modulating agents. An increase or decrease in SPL levels may be measured by evaluating the level of SPL mRNA and/or protein in the presence and absence of candidate modulating agent. For example, an antisense modulating agent may be evaluated by assaying the effect on SPL levels. Suitable cells for use in such assays include the breast cancer cell lines MCF-7 (ATCC Accession Number HTB-22) and MDA-MB-231 (ATCC Accession Number HTB-26). A candidate modulator may be tested by transfecting the cells with a polynucleotide encoding the candidate and evaluating the effect of expression of the polynucleotide on SPL levels. Alternatively, the cells may be

contacted with a candidate modulator, typically in an amount ranging from about 10 nM to about 10 mM. A candidate that results in a statistically significant change in the level of SPL mRNA and/or protein is a modulating agent.

Alternatively, or in addition, a candidate modulating agent may be tested for the ability to inhibit or increase SPL activity, using an *in vitro* assay as described herein (see Van Veldhoven and Mannaerts, *J. Biol. Chem.* 266:12502-07, 1991) that detects the degradation of labeled substrate (*i.e.*, sphingosine-1-phosphate, or a derivative thereof). Briefly, a solution (*e.g.*, a cellular extract) containing an SPL polypeptide (*e.g.*, 10 nM to about 10 mM) may be incubated with a candidate modulating agent (typically 1 nM to 10 mM, preferably 10 nM to 1 mM) and a substrate (*e.g.*, 40  $\mu$ M) at 37°C for 1 hour in the presence of, for example, 50 mM sucrose, 100 mM K-phosphate buffer pH 7.4, 25 mM NaF, 0.1% (w/v) Triton X-100, 0.5 mM EDTA, 2 mM DTT, 0.25 mM pyridoxal phosphate. Reactions may then be terminated and analyzed by thin-layer chromatography to detect the formation of labeled fatty aldehydes and further metabolites. A modulating agent (*e.g.*, an antibody) that increases SPL activity results in a statistically significant increase in the degradation of sphingosine-1-phosphate, relative to the level of degradation in the absence of modulating agent. Such modulating agents may be used to increase SPL activity in a cell culture or a mammal, as described below.

A modulating agent may additionally comprise, or may be associated with, a targeting component that serves to direct the agent to a desired tissue or cell type. As used herein, a "targeting component" may be any substance (such as a compound or cell) that, when linked to a compound enhances the transport of the compound to a target tissue, thereby increasing the local concentration of the compound. Targeting components include antibodies or fragments thereof, receptors, ligands and other molecules that bind to cells of, or in the vicinity of, the target tissue. Known targeting components include hormones, antibodies against cell surface antigens, lectins, adhesion molecules, tumor cell surface binding ligands, steroids, cholesterol, lymphokines, fibrinolytic enzymes and other drugs and proteins that bind to a desired target site. In particular, anti-tumor antibodies and compounds that bind to an estrogen receptor may serve as targeting components. An antibody employed in the present invention may be an intact

(whole) molecule, a fragment thereof, or a functional equivalent thereof. Examples of antibody fragments are F(ab')2, -Fab', Fab and F[v] fragments, which may be produced by conventional methods or by genetic or protein engineering. Linkage may be via any suitable covalent bond using standard techniques that are well known in the art. Such linkage is generally covalent and 5 may be achieved by, for example, direct condensation or other reactions, or by way of bi- or multi-functional linkers.

For *in vivo* use, a modulating agent as described herein is generally incorporated into a pharmaceutical composition prior to administration. A pharmaceutical composition comprises one or more modulating agents in combination with a physiologically acceptable carrier. To prepare a pharmaceutical composition, an effective amount of one or more modulating agents is mixed with any pharmaceutical carrier(s) known to those skilled in the art to be suitable for the particular mode of administration. A pharmaceutical carrier may be liquid, semi-liquid or solid. Solutions or suspensions used for parenteral, intradermal, subcutaneous or topical application may include, for example, a sterile diluent (such as water), saline solution, 10 fixed oil, polyethylene glycol, glycerine, propylene glycol or other synthetic solvent; antimicrobial agents (such as benzyl alcohol and methyl parabens); antioxidants (such as ascorbic acid and sodium bisulfite) and chelating agents (such as ethylenediaminetetraacetic acid (EDTA)); buffers (such as acetates, citrates and phosphates). If administered intravenously, suitable carriers include physiological saline or phosphate buffered saline (PBS), and solutions 15 containing thickening and solubilizing agents, such as glucose, polyethylene glycol, polypropylene glycol and mixtures thereof. In addition, other pharmaceutically active ingredients (including other anti-cancer agents) and/or suitable excipients such as salts, buffers and stabilizers may, but need not, be present within the composition.

A modulating agent may be prepared with carriers that protect it against rapid 20 elimination from the body, such as time release formulations or coatings. Such carriers include controlled release formulations, such as, but not limited to, implants and microencapsulated delivery systems, and biodegradable, biocompatible polymers, such as ethylene vinyl acetate,

polyanhydrides, polyglycolic acid, polyorthoesters, polylactic acid and others known to those of ordinary skill in the art.

Administration may be achieved by a variety of different routes, including oral, parenteral, nasal, intravenous, intradermal, subcutaneous or topical. Preferred modes of administration depend upon the nature of the condition to be treated or prevented. An amount that, following administration, inhibits, prevents or delays the progression and/or metastasis of a cancer is considered effective. Preferably, the amount administered is sufficient to result in regression, as indicated by 50% mass or by scan dimensions. The precise dosage and duration of treatment is a function of the disease being treated and may be determined empirically using known testing protocols or by testing the compositions in model systems known in the art and extrapolating therefrom. Controlled clinical trials may also be performed. Dosages may also vary with the severity of the condition to be alleviated. A pharmaceutical composition is generally formulated and administered to exert a therapeutically useful effect while minimizing undesirable side effects. The composition may be administered one time, or may be divided into a number of smaller doses to be administered at intervals of time. For any particular subject, specific dosage regimens may be adjusted over time according to the individual need.

As an alternative to direct administration of a modulating agent, a polynucleotide encoding a modulating agent may be administered. Such a polynucleotide may be present in a pharmaceutical composition within any of a variety of delivery systems known to those of ordinary skill in the art, including nucleic acid, bacterial and viral expression systems, and colloidal dispersion systems such as liposomes. Appropriate nucleic acid expression systems contain the necessary DNA sequences for expression in the patient (such as a suitable promoter and terminating signal, as described above). The DNA may also be "naked," as described, for example, in Ulmer et al., *Science* 259:1745-49, 1993.

Various viral vectors that can be used to introduce a nucleic acid sequence into the targeted patient's cells include, but are not limited to, vaccinia or other pox virus, herpes virus, retrovirus, or adenovirus. Techniques for incorporating DNA into such vectors are well known to those of ordinary skill in the art. Another delivery system for polynucleotides is a colloidal

dispersion system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The preparation and use of liposomes is well known to those of ordinary skill in the art.

5 Within certain aspects of the present invention, one or more modulating agents may be used to modulate SPL expression and/or activity *in vitro*, in a cell or in a mammal. *In vitro*, an SPL polypeptide may be contacted with a modulating agent that increases or decreases SPL activity (e.g., certain antibodies). For use within a cell or a mammal, such modulation may be achieved by contacting a target cell with an effective amount of a modulating agent, as described herein. Administration to a mammal may generally be achieved as described above.

10 As noted above, increase of SPL expression and/or activity provides a method for inhibiting the growth (*i.e.*, proliferation) of a cancer cell, either in culture or in a mammal afflicted with cancer. *In vivo*, such increase may also be used to inhibit cancer development, progression and/or metastasis. Accordingly, one or more modulating agents as provided herein 15 may be administered as described above to a mammal in need of anti-cancer therapy. Patients that may benefit from administration of a modulating agent are those afflicted with cancer. Such patients may be identified based on standard criteria that are well known in the art. Within preferred embodiments, a patient is afflicted with breast cancer, as identified based on tissue biopsy and microscopic evaluation, using techniques well known in the art. In particular, 20 patients whose tumor cells contain a tissue-specific deletion and/or alteration within an endogenous SPL gene may benefit from administration of a modulating agent, as provided herein.

25 Within other aspects, the present invention provides methods and kits for diagnosing cancer and/or identifying individuals with a risk for metastasis that is higher or lower than average. It has been found, within the context of the present invention, that certain human tumor cells contain an altered SPL gene. In particular, certain brain tumor cells contain a deletion of amino acid residues 354 to 433 of the human SPL sequence set forth in SEQ ID NO:8 (cDNA and amino acid sequence of the SPL containing the deletion are set forth in SEQ ID

NOs:9 and 10, respectively). Specific alterations present in other tumor cells, such as breast tumor cells, may be readily identified using standard techniques, such as PCR. Alterations that may be associated with a particular tumor include amino acid deletions, insertions, substitutions and combinations thereof. Methods in which the presence or absence of such an alteration is determined may generally be used to detect cancer and to evaluate the prognosis for a patient known to be afflicted with cancer.

To detect an altered SPL gene, any of a variety of well-known techniques may be used including, but not limited to, PCR and hybridization techniques. Any sample that may contain cancerous cells may be assayed. In general, suitable samples are tumor biopsies. Within a preferred embodiment, a sample is a breast tumor biopsy.

Kits for diagnosing or evaluating the prognosis of a cancer generally comprise reagents for use in the particular assay to be employed. In general, a kit of the present invention comprises one or more containers enclosing elements, such as primers, probes, reagents or buffers, to be used in an assay. For example, a kit may contain one or more polynucleotide primers or probes comprising at least 15 nucleotides complementary to a polynucleotide encoding SPL. In certain embodiments, the primers or probes comprise at least 10, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 nucleotides, and preferably at least 150 or 200 nucleotides, complementary to an SPL mRNA or to a polynucleotide encoding SPL. Such probe(s) may be used to detect an altered SPL gene by hybridization. For example, a kit may contain one probe that hybridizes to a region of an SPL gene that is not generally altered in tumors (a control) and a second probe that hybridizes to a region commonly deleted in breast cancer. A sample that contains mRNA that hybridizes to the first probe, and not to the second (using standard techniques) contains an altered SPL gene. Suitable control probes include probes that hybridize to a portion of the SPL gene outside of the commonly deleted region encoding amino acid residues 354 to 433; suitable probes for an altered region include probes that hybridize to a portion of the SPL gene that encodes amino acid residues 354 to 433. Alternatively, a kit may comprise one or more primers for PCR analyses, which may be readily designed based upon the sequences provided herein by those of ordinary skill in the art. Optionally, a kit may further

10  
0000000000  
15  
20  
25  
30  
35  
40  
45  
50  
55  
60  
65  
70  
75  
80  
85  
90  
95  
0000000000

comprise one or more solutions, compounds or detection reagents for use within an assay as described above.

In a related aspect of the present invention, kits for detecting SPL are provided. Such kits may be designed for detecting the level of SPL or nucleic acid encoding SPL within a sample, or may detect the level of SPL activity as described herein. A kit for detecting the level of SPL, or nucleic acid encoding SPL, typically contains a reagent that binds to the SPL protein, DNA or RNA. To detect nucleic acid encoding SPL, the reagent may be a nucleic acid probe or a PCR primer. To detect SPL protein, the reagent is typically an antibody. The kit may also contain a reporter group suitable for direct or indirect detection of the reagent as described above.

Within further aspects, the present invention provides transgenic mammals in which SPL activity is reduced, compared to a wild-type animal. Such animals may contain an alteration, insertion or deletion in an endogenous SPL gene, or may contain DNA encoding a modulating agent that modulates expression or activity of an SPL gene. In certain aspects, such animals may contain DNA encoding a modulating agent that increases expression or activity of an SPL gene. Transgenic animals may be generated using techniques that are known to those of ordinary skill in the art. For example, a transgenic animal containing an insertion or deletion in the coding region for the SPL gene may be generated from embryonic stem cells, using standard techniques. Such stem cells may be generated by first identifying the full genomic sequence of the gene encoding the SPL, and then creating an insertion or deletion in the coding region in embryonic stem cells. Alternatively, appropriate genetically altered embryonic stem cells may be identified from a bank. Using the altered stem cells, hybrid animals may be generated with one normal SPL gene and one marked, abnormal gene. These hybrids may be mated, and homozygous progeny identified.

Transgenic animals may be used for a variety of purposes, which will be apparent to those of ordinary skill in the art. For example, such animals may be used to prepare cell lines from different tissues, using well known techniques. Such cell lines may be used, for example, to evaluate the effect of the alteration, and to test various candidate modulators.

The invention further provides *Drosophila melanogaster* animal models that exhibit a flightless phenotype, where the phenotype results from the disruption of an endogenous SPL gene as described in greater detail below. By flightless phenotype is meant that the subject non-mammalian animal models spontaneously develop a reduced number of muscle fibers comprising the dorsal longitudinal muscles (DLM) and have compensatory hypertrophy in the remaining fibers. In certain aspects, the non-mammalian animal model of the present invention may also demonstrate abnormal developmental patterning of thoracic muscles of the T2 segment. In a preferred embodiment, the above phenotypes result in an inability to fly. The subject non-mammalian animal models, within a preferred embodiment, demonstrate altered activity of the endogenous SPL. In a particularly illustrative embodiment, said *D. melanogaster* animal models have decreased activity of endogenous SPL.

10  
15  
20  
25

Within further aspects, the present invention provides mutant strains of *Drosophila melanogaster*. In a preferred embodiment, the strain contains a mutation in the SPL gene. In a further embodiment of the present invention the *D. melanogaster* strain are heterozygous for a P-element transposon which sits in the coding region of the gene encoding the SPL protein set forth in SEQ ID NO:16. In a preferred embodiment, the flies are homozygous insertional mutants in the coding region of the gene encoding the SPL protein set forth in SEQ ID NO:16. In yet a further embodiment of the present invention, the homozygous mutant strain of fly has a flightless phenotype. In certain embodiments, the mutant flies have a reduced number of muscle fibers comprising the dorsal longitudinal muscles and have compensatory hypertrophy in the remaining fibers. In certain aspects, the mutant flies of the present invention may also demonstrate abnormal developmental patterning of thoracic muscles of the T2 segment.

Flies heterozygous for a P-element transposon which sits in the coding region of the SPL gene or genes and disrupts production of SPL proteins may be obtained from the *Drosophila* Genome Project. Homozygous insertional mutants can be made, using techniques known in the art, by genetically crossing and evaluating progeny for the presence of homozygous insertional mutants (based on presence of rosy eye color, encoded by a recessive marker carried on the P-element). Expression of the SPL gene can be evaluated using any number of assays

known to the skilled artisan, for example, by Northern blot analysis. To determine the SPL function of each genotype, +/+, +/- and -/- flies may be homogenized using standard techniques and whole extracts can be assayed for SPL activity using assays as described herein. The transposon can be mobilized by crossing SPL mutant flies with flies carrying an actively transcribed transposase gene, which should cause the P-element to be excised in the chromosomes of both somatic cells and in the germline. Germline transposon loss is heritable and can be identified in progeny by virtue of eye color. Progeny which lost both the transposase gene and the P-element can then be isolated and the restored SPL allele can be homozygosed.

Mutations in *Drosophila melanogaster* as described herein which permanently block expression of a functional protein can be created in several ways, such as with P-element transposon insertions or chemical or radiation induced mutagenesis. Exemplary strains of mutant flies are available through the *Drosophila* Genome Project, at the University of California at Berkeley (Adams, M. et al 2000. The genome sequence of *Drosophila melanogaster*. *Science*. 287:2185-2195.). Alternatively, insertional mutant of interest may be obtained by using local hop strategies essentially as described in Tower, J. et al (Tower, J., et al. 1993. Preferential transposition of *Drosophila* P elements to nearby chromosomal sites. *Genetics*. 133:347-359.), hereby incorporated by reference in its entirety. Transposons can be mobilized by crossing in a transposase gene, followed by crossing the transposase back out (reintroducing genetic stability). Mutant flies can be identified using techniques known to those of skill in the art. For example, mutant flies can be identified by probing Southern blots prepared from extracts from flies generated in the screen using the target gene as probe. Subsequently, crosses can be performed to introduce a mutant allele of interest, (e.g. SPL) and generate homozygosity at both mutant alleles (e.g. SPL *and* new transposon integration sites). Mutants can be screened for a phenotype of interest, for example the ability to restore flight to an SPL mutant when the mutated allele is homozygous (predicting a recessive phenotype).

In one aspect of the present invention, fly genetic manipulation may entail mating or “crossing” of flies and selection for or against progeny expressing various phenotypic markers. Exemplary techniques for fly genetic manipulation of the present invention are known in

the art and are described, for example in, Ashburner, M., and J. Roote. 2000. Laboratory culture of *Drosophila*. In *Drosophila Protocols*. W. Sullivan, M. Ashburner, and R. Hawley, editors. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 585-600. Phenotypic markers may be used to identify the inheritance of chromosomes, engineered transposable elements, or 5 transposase genes used to facilitate their mobilization. Marker mutations affecting eye color, bristle shape, wing morphology and cuticle pigmentation, for example, may be employed in the crosses for the mutant flies of the present invention. Within one aspect of the present invention, it may be desirable to select the individuals which contain a collection of markers indicating the desired genotype. In another aspect of the present invention, balancer chromosomes may be used 10 to create the ability to identify recessive mutations present in the heterozygous state. Balancer chromosomes may be employed to prevent homologous recombination during meiotic prophase in females. The presence of both dominant and recessive lethal markers allows one to determine the presence or absence of the balancer chromosomes and simultaneously to follow the homologous chromosomes, which may themselves not contain a dominant marker. One 15 particularly illustrative cross of the present invention is to eliminate the P-element insertion in the *Drosophila* SPL gene and establish phenotypic reversion, as described herein in the Examples.

Selective markers to allow for selection of mutant flies is provided for in the present invention. Exemplary selective markers of the present invention may comprise a wild 20 type rosy (*ry*<sup>+</sup>) allele carried on the transposon to allow for selection for or against the stable transposon. Introduction of an active transposase is selected for by presence of the dominant marker, Stubble (short bristle phenotype) in the first cross, and is selected against to identify progeny which have lost the transposase, restoring genetic stability in the second cross. Other 25 illustrative markers include Curly O (CyO) which is lethal when present in two copies, allowing selection for heterozygotes containing the CyO balancer and another allele of interest originally containing the transposon (e.g., SPL). By selecting against rosy eye color, progeny in which the transposon has been excised from the locus of interest, e.g., SPL, can be identified. Expansion of this "reverted" allele in the population can be achieved in the third cross, and the desired allele

can be homozygosed in the final cross, to determine whether restoration of the intact allele of interest, for example SPL, is associated with a desired phenotype of interest, such as restoration of flight.

In another aspect of the present invention, transgenic flies can be created using P-  
5 elements to overexpress or misexpress proteins of interest, such as SPL. In one embodiment of  
the invention, GAL4-mediated ectopic gene expression is employed, essentially as described (van  
Roessel, P., and A. Brand. 2000. GAL4-mediated ectopic gene expression in *Drosophila*. In  
*Drosophila Protocols*. W. Sullivan, M. Ashburner, and R. Hawley, editors. Cold Spring Harbor  
Laboratory Press, Cold Spring Harbor, NY. 439-448.). The GAL4 protein is a yeast transcription  
10 factor capable of activating transcription of *Drosophila* genes which have been engineered to  
contain upstream sequences recognized by the GAL4 protein. Various mutants can be created  
with a gene of interest expressed in specific tissue distributions, a construct containing the gene  
of interest (reporter) under regulation of a GAL4 containing promoter is introduced into embryos,  
and a genetic marker allows identification of progeny containing this construct. Illustrative  
15 GAL4 containing promoters include, but are not limited to, pUAS. The use of embryos of a  
strain containing an active P-transposase increases the efficiency of transgene integration,  
although many of the embryos die. These progeny can then be crossed to various available lines  
containing GAL4 transgenes (driver) expressed under control of tissue-specific promoters. In  
one aspect of the present invention, GAL4 driver constructs which allow expression during  
20 embryogenesis are used.

The following Examples are offered by way of illustration and not by way of  
limitation.

## EXAMPLES

## Example 1

Isolation and Characterization of SPL cDNA from Yeast

5 This Example illustrates the preparation of an *S. cerevisiae* cDNA molecule encoding an endogenous SPL polypeptide.

Wild-type yeast cells (SGP3 (Garrett and Broach, *Genes and Dev.* 3:1336-1348, 1989); *leu2-3,112 trp1 ura3-52 his3 ade8 ras1::HIS3*) were transformed with a yeast genomic library carried on the pRS202 high-copy shuttle vector (Sikorski and Heiter, *Genetics* 122:19-27, 1989) containing a selectable nutritional marker (*URA3*). pRS202 is a modified version of the pRS306 vector, into which a 2 micron plasmid piece was inserted. Inserts from this library are approximately 6-8 kb in length. Wild type yeast were transformed with the high copy library as described by Ito et al., *J. Bact.* 153:163-68, 1983, selected for uracil prototrophy (*i.e.*, the ability to grow on medium lacking uracil), and transformants were pooled and replated at a concentration of  $10^6$  cells per plate onto 1 mM D-*erythro*-sphingosine plates.

15 Six transformants which grew large colonies on 1mM D-*erythro*-sphingosine plates were grown in selective medium, and control SGP3 colonies were grown in minimal medium, at 30°C until saturated. Absorbance at 660 nm was used to correct for small variations in cell concentration between cultures. Serial dilutions were performed, and cells were template-  
20 inoculated onto 1 mM D-*erythro*-sphingosine plates and incubated at 30°C for 48 hours.

25 The most highly represented insert, 13-1, was subcloned and sequenced, and named *BST1* (bestower of sphingosine tolerance; GenBank accession number U51031; *Saccharomyces cerevisiae* genome database accession number YDR294C). The *BST1* nucleotide sequence encodes a previously unknown predicted protein of 65,523 kilodaltons and 589 amino acids in length. This sequence is 23% identical to *gadA* and *gadB*, two nearly identical *E. coli* genes encoding glutamate decarboxylase (GAD), a pyridoxal-5'-phosphate-dependent enzyme which catalyzes synthesis of the neurotransmitter  $\gamma$ -amino butyric acid. *BST1* has been localized to *S. cerevisiae* chromosome 4. The DNA sequence of *BST1* is provided in SEQ ID NO:1, which encodes the amino acid sequence set forth in SEQ ID NO:2.

To explore the function of *BST1*, a deletion strain was created through homologous recombination using a *NEO* selectable marker (Wach et al., *Yeast* 10:1793-1808, 1994). Genomic *BST1* was replaced with kanMX (Wach et al., *Yeast* 10:1793-1808, 1994), which confers resistance to G418. Disruption was confirmed using PCR amplification of 5 genomic DNA from G418 resistant clones, using primers to genomic sequence just 5' and 3' to the region replaced by the disruption. Deletion of *BST1* and all subsequent biological studies were performed in both SGP3 and in JK93d (Hietman et al., *Proc. Natl. Acad. Sci. USA* 88:1948-52, 1991); *ura3-52 leu2-3,112 his4 trp1 rme1*). Heterozygous diploids were sporulated, and spores segregated 2:2 for G418 resistance. Both G418 resistant and sensitive progeny were 10 viable, indicating that *BST1* is not an essential gene.

Analysis of GAD activity in cytosolic extracts from wild type, *BST1* overexpression and *bst1Δ* strains indicated that *BST1* does not encode the *S. cerevisiae* homologue of GAD. However, deletion of *BST1* was associated with severe sensitivity to D-*erythro*-sphingosine. Concentrations as low as 10 μM sphingosine completely inhibited growth of *bst1Δ* 15 strains but had no effect on the viability of wild type cells. In comparison to the control strain, the *bst1Δ* strain also demonstrated greater sensitivity to 100 μM phytosphingosine, the long chain base endogenous to *S. cerevisiae*. No difference between the growth of wild type and *BST1* overexpression strains on phytosphingosine, which is only minimally toxic to wild type cells at this concentration, was observed.

20 To determine whether differences in sphingosine uptake or metabolism were responsible for these sensitivity differences, *BST1* wild type, overexpression and *bst1Δ* strains were exposed to [C3-<sup>3</sup>H]labeled sphingosine (American Radiolabeled Chemical, Inc., St. Louis, MO), washed in sterile water and subjected to Bligh-Dyer extractions (Bligh and Dyer, *Can. J. Biochem. Physiol.* 37:911-17, 1959). There were no major differences in sphingosine recovery 25 among the three strains. However, the aqueous phase from the *bst1Δ* strain contained a ten-fold increase in radioactivity over that of control and *BST1* overexpression strains. Thin layer chromatography (TLC) analysis of the lipid fractions in butanol:acetic acid:water (3:1:1) revealed a sphingosine band which appeared equivalent in each strain.

Radioactive sphingosine-1-phosphate (S-1-P) was also observed in the extracts from the *bst1Δ* strain, but not in the wild type or *BST1* overexpression strains. This compound accumulated rapidly, reaching a plateau by 60 minutes. Three separate TLC conditions were used to confirm the presence of S-1-P. These conditions, along with the resulting RF values, are 5 shown below:

butanol:water:acetic acid (3:1:1)	.47
chloroform:methanol:water (60:35:8)	.22
chloroform:methanol:water:acetic acid (30:30:2:5)	.33

Hyperaccumulation of S-1-P and hypersensitivity to D-*erythro*-sphingosine suggests a failure to metabolize S-1-P, indicating that *BST1* is a yeast SPL. To confirm this identification, lyase activity in *BST1* wild type, overexpression and deletion strains were evaluated as described by Veldhoven and Mannaerts, *J. Biol. Chem.* 266:12502-07, 1991, using unlabeled D-*erythro*-dihydrosphingosine-1-phosphate (Biomol, Plymouth Meeting, PA) and D-*erythro*-dihydrosphingosine [4,5-<sup>3</sup>H]1-phosphate (American Radiolabeled Chemicals, Inc., St. Louis, MO). Specific activity was 100 mCi/mmol. SPL activity was found to correlate with *BST1* expression, confirming *BST1* to be the yeast homologue of sphingosine-1-phosphate lyase.

These results indicate that *BST1* is a yeast SPL, and that SPL catalyzes a rate-limiting step in sphingolipid catabolism. Regulation of SPL activity may therefore result in regulation of intracellular S-1-P levels.

25 Example 2  
Isolation and Characterization of SPL cDNA from *C. elegans* and Mouse

This Example illustrates the identification of endogenous SPL cDNAs from *C. elegans* and *Mus musculus*.

Comparison of the yeast *BST1* sequence to sequences within the GenBank database identified a full length gene from *C. elegans* that was identified during the systematic sequencing of the *C. elegans* genome. This cDNA sequence is set forth in SEQ ID NO:3 and was found to encode SPL, the sequence set forth in SEQ ID NO:4. This and other DNA 5 homology searches described herein were performed via the National Center for Biotechnology Information website using BLAST search program.

Using both *S. cerevisiae* and *C. elegans* SPL sequences to search the EST database, an expressed sequence tag from early embryonic cells of the mouse (day 8 embryo, strain C57BL/6J) was identified. The cDNA clone containing this putative mouse SPL was 10 purchased from Genome Systems, Inc (St. Louis, MO). Completion of the full length cDNA sequence revealed an 1707 bp open reading frame. This mouse sequence showed significant homology to *BST1* and to other pyridoxal phosphate-binding enzymes such as glutamate decarboxylase, with greatest conservation surrounding the predicted pyridoxal phosphate-binding lysine. Since the two genes encoding mouse glutamate decarboxylase have been identified 15 previously, and the identified sequence was unique and had no known function, it was a likely candidate mouse SPL gene.

To confirm the SPL activity of the mouse gene, a two step process was undertaken. First, the sequence was cloned into the high-copy yeast expression vector, pYES2 (Invitrogen, Inc., Carlsbad, CA), in which the gene of interest is placed under control of the yeast 20 GAL promoter and is, therefore, transcriptionally activated by galactose and repressed by glucose. pYES2 also contains the *URA3* gene (which provides transformants the ability to grow in media without uracil) and an ampicillin resistance marker and origin of replication functional in *E. coli*.

The expression vector containing the full-length mouse SPL gene was then 25 introduced into the yeast *bst1Δ* strain which, as noted above, is extremely sensitive to D-*erythro*-sphingosine, as a result of metabolism of sphingosine to S-1-P. S-1-P cannot be further degraded in the absence of SPL activity and overaccumulates, causing growth inhibition. Transformation was performed using the lithium acetate method (Ito et al., *J. Bact.* 153:163-68, 1983).

Transformants were grown on medium containing 20g/L galactose and selected for uracil prototrophy.

Transformants were then evaluated for sphingosine resistance. Strains of interest were grown to saturation in liquid culture for 2-3 days. They were then resuspended in minimal medium, placed in the first row of a 96-well plate and diluted serially from 1:2 to 1:4000 across the plate. The cultures were then template inoculated onto a control plate (YPD) and a plate containing minimal synthetic media supplemented with 50  $\mu$ M D-*erythro*-sphingosine (Sigma Chemical Co., St. Louis, MO) and 0.0015% NP40 (Sigma Chemical Co.). At this concentration of NP40, no effects on cell viability were observed. Plates were incubated at 30°C for two days and assessed visually for differences in growth. Transformants containing the mouse SPL gene were resistant to sphingosine present in galactose-containing plates. A strain transformed with vector alone remained sensitive to sphingosine. Therefore, the mouse SPL gene was capable of reversing the sphingosine-sensitive phenotype of a yeast *bst1* $\Delta$  strain.

In order to determine whether the mouse SPL gene was able to restore biochemical SPL activity to the *bst1* $\Delta$  strain, the untransformed *bst1* $\Delta$  strain, and the *bst1* $\Delta$  strain transformed with pYES2 containing either *BST1* or the putative mouse SPL gene were grown to exponential phase ( $A_{600}=1.0$ ) in either minimal (JS16) or uracil medium containing galactose as a carbon source. Whole cell extracts were prepared from each strain as described above, adjusted for protein concentration, and evaluated for sphingosine phosphate lyase activity as described above, using  $^3$ H-dihydrosphingosine-1-phosphate (American Radiolabeled Chemicals, Inc., St. Louis, MO). Qualitative analysis of product was performed by autoradiography. Quantitative measurement was performed by scraping TLC plates and determining radioactivity present using a standard scintillation counter.

The results of the sphingosine phosphate lyase assays shown that expression of both the yeast and mouse sequences restored SPL activity to the *bst1* $\Delta$  strain, whereas vector alone had no effect, confirming the identity of the mouse sequence as SPL.

To determine whether the expression of the mouse SPL transcript coincided with previously reported tissue-specific SPL activity in the mouse, total RNA was obtained from a

variety of mouse tissues and probed with the complete mouse SPL cDNA sequence. Northern analysis was performed as described by Thomas, *Proc. Natl. Acad. Sci. USA* 77:5201, 1980, using a full length mouse SPL cDNA probe labeled by random labeling technique (Cobianchi and Wilson, *Meth. Enzymol.* 152:94-110, 1987). This analysis revealed a pattern of expression 5 consistent with the known SPL activity in various mouse tissues, providing further confirmation that this sequence encodes mouse SPL.

Example 3  
Isolation and Characterization of Human SPL cDNA

10 This Example illustrates the identification of an endogenous human cDNA. An EST database was searched using the mouse SPL sequence described herein. Two distinct EST sequences having strong homology to the mouse sequence were identified from human sources. One of these sequences corresponded to the C-terminus, and the other corresponded to the N-terminus. Primers were designed based on these sequences, and a DNA 15 fragment was amplified by PCR from a human expression library made from human glioblastoma multiforme tissue RNA. The fragment was sequenced and was shown to contain a deletion, so the primers were used to amplify the gene from human fibroblast RNA. This gene has the sequence provided in SEQ ID NO:7 and encodes the polypeptide sequence provided in SEQ ID NO:8. The cDNA and amino acid sequences of the SPL containing the deletion are set 20 forth in SEQ ID NOs:9 and 10, respectively.

Example 4  
Isolation and Characterization of *C. Elegans* SPL cDNA

25 This Example illustrates the identification of a cDNA molecule encoding a primary *C. elegans* sphingosine phosphate lyase.

The human SPL cDNA sequence was used to screen the ACEdb *C. elegans* genome database. A potential *C. elegans* open reading frame of unknown function present on YAC Y66H1B showed substantial (40%) homology to yeast, human and mouse SPL cDNA sequences. To clone this sequence, a coupled reverse transcriptase/polymerase chain reaction

was performed using the Access RT-PCR system (see below). Template was *C. elegans* total RNA, and primers were:

5'-GAGGAATTCATGGATTGGTTAACGACACAAACCG-3'

5'-AGCCTCGAGTTAATTAGAAGTTGAAGGTGGAGC-3'

This resulted in a DNA fragment cSPL2, which was ligated into the yeast expression vector pYES2, obtained from Invitrogen. Inc. (Carlsbad, CA). Genes expressed using this system are regulated under the control of the GAL promoter, which allows expression in the presence of galactose and not in the presence of glucose. The nucleotide sequence of cSPL2 is set forth in SEQ ID NO:12 , with the encoded amino acid sequence set forth in SEQ ID NO:11

cSPL2 was further analyzed for its ability to complement the sphingosine sensitive phenotype of a yeast *dpl1* mutant, the previously described yeast strain JS16 which contains a large deletion in *DPL1*, the *S. cerevisiae* sphingosine phosphate lyase gene (Zhou and Saba, *Biochem Biophys Res Commun* 242:502-507, 1998). Transformation of JS16 with pYES2 or the *C. elegans* SPL-pYES2 construct was performed by the lithium acetate method (Ito et al., *J. Bact.* 153:163-168, 1983). Transformants were selected for uracil prototrophy and evaluated for sphingosine resistance using the dilutional assay described by Zhou and Saba, *Biochem Biophys Res Commun* 242:502-507, 1998. Cells were grown in minimal or uracil<sup>-</sup> media containing either 20g glucose or galactose per liter, as indicated. D-*erythro*-sphingosine and NP40 were obtained from Sigma Chemical Company (St. Louis, MO).

The results demonstrate that cSPL2 convincingly complemented the yeast mutant, restoring enzyme activity. In each plate, yeast were grown to saturation in overnight liquid cultures, spun down, resuspended in 200 microliters of water and dispensed into the first (left-most) well of each horizontal row. Yeast were then further diluted into sterile water, so the second well was 1:2, third well was 1:4, fourth well was 1:40, fifth was 1:400 and sixth was 1:4000 dilution from the original on the left. The toxicity of sphingosine is cell number dependent, because it disperses itself in cell membranes. Therefore, the concentration of sphingosine in the plate is not the only thing affecting toxicity, and these dilutional assays show

differences in tolerance/sensitivity. So, a strain which can grow in the sixth row is about 4,000 times more resistant to sphingosine than one which can grow only in the first row.

The mutant yeast strain containing cSPL2 also demonstrated substantial SPL activity. The sphingosine phosphate lyase assay used whole cell extracts of yeast containing either pYES2 vector alone or (cSPL2) *C.elegans* SPL-pYES2. Extracts were prepared as described by Saba et al., *J Biol Chem* 272:26087, 1997. SPL activity was determined essentially as described, using  $^3\text{H}$ -dihydrosphingosine-1-phosphate substrate (see Zhou and Saba, *Biochem Biophys Res Commun* 242:502-507, 1998). Substrate for SPL assay ( $^3\text{H}$ -dihydrosphingosine-1-phosphate) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Access RT-PCR system was obtained from Promega Corp. (Madison, WI).

Enzyme activity in (cSPL2) *C. elegans* SPL-pYES2 was appreciably greater than that of the vector control. These results indicate that cSPL2 encodes the primary *C. elegans* SPL.

## Example 5

### Developmental Defects Induced by RNA Interference in *C. elegans*

In order to determine the effect of blocking cSPL2 expression on the development of *C. elegans*, RNA interference studies were undertaken. The cSPL2 cDNA was cloned into pBluescript such that the insert was flanked by the T3 and T7 promoter regions. RNA complementary to each strand was synthesized from these promoters using an *in vitro* transcription kit (Promega, Madison, WI). The two strands were annealed to make double stranded RNA (dsRNA) and injected into the distal gonads of 12 wild-type (N2 Bristol) young adult *C. elegans* hermaphrodites. As controls, uninjected hermaphrodites as well as hermaphrodites injected with a dsRNA that does not produce a visible phenotype were handled in parallel. Eight hours after injection, each hermaphrodite was transferred to a fresh culture plate and 12 hour cohorts of F1 progeny were established. Progeny were observed daily with a dissecting microscope until most animals reached adulthood and the culture plates became too crowded with F2 progeny. Compared to control F1s, animals inheriting cSPL-2 dsRNA developed slowly, moved sluggishly, were thin and pale, and did not pump food actively. These

5 animals reach adulthood approximately 24 hours later than controls. Adult hermaphrodites that inherited cSPL-2 dsRNA were markedly different from controls especially in the gonad and uterus. Control animals had abundant nuclei in the distal gonad and a row of developing oocytes in the proximal gonad. Affected hermaphrodites had poorly developed distal gonads with fewer nuclei. Control adults had embryos of progressive stages of development in the uterus, whereas the number of developing oocytes in the proximal gonad of affected hermaphrodites was reduced. The embryos in the uterus of affected progeny were also abnormal. Those near the vulva were at late developmental stages indicating a defect in egg laying. There was not a uniform progression of developmental stages in adjacent embryos suggesting a defect in ovulation or development, and some of the embryos showed abnormal patterns of cell division. 10 In summary, inhibition of *C. elegans* SPL expression through the use of RNA interference leads to poor feeding, developmental abnormalities and impaired fertility in the progeny. These results suggest that SPL is an essential gene in *C. elegans*.

### Example 6

## Isolation and Characterization of SPL cDNA from *Drosophila melanogaster*

In order to seek out the *Drosophila melanogaster* SPL cDNA and genomic sequence, the *D. melanogaster* genomic database was searched for sequences which demonstrated significant homology to human SPL cDNA. This led to identification of two full-length cDNA clones (LP04413 and GH3783) which were confirmed by sequence and restriction analysis. The two clones are predicted based on alternative 5' exon usage and may be expressed in different subcellular locations. The predicted *Drosophila melanogaster* SPL is located on the right arm of chromosome II, position 53F8-12. The cDNA sequence for *Drosophila melanogaster* SPL is set forth in SEQ ID NO:15 and encodes the SPL protein set forth in SEQ ID NO:16. The *Drosophila* SPL predicted protein sequence set forth in SEQ ID NO:16 is 49%, 49% and 43% identical to human, mouse and yeast SPL protein sequences, respectively.

In order to evaluate whether these clones contained a functional SPL gene, they were recloned into the yeast expression vector, pYES2, and this construct was transformed into a

*dpl1Δ* strain. Expression of clones containing the potential *Drosophila melanogaster* SPL fully complement the *dpl1Δ* strain's sensitivity to 50 µM D-*erythro*-sphingosine. Further, whole cell extracts of *dpl1* strains containing either pYES2-LP04413 or pYES2-GH3783 demonstrate restoration of SPL enzyme activity to wild type levels or greater, although not as high as a *DPL1* overexpressing strain (DPL OE).

#### Example 7

##### Generation and Characterization of SPL transposon mutant *D. melanogaster*

Flies heterozygous for a P-element transposon which sits in the coding region of both of the above transcripts described in Example 6 and presumably disrupts both SPL proteins were obtained from the *Drosophila* Genome Project. These flies were genetically crossed using techniques well known to ordinarily skilled artisans, and progeny were evaluated for the presence of homozygous insertional mutants (based on presence of rosy eye color, encoded by a recessive marker carried on the P-element). Northern blot analysis from wild type and SPL insertional mutant flies indicated that no SPL gene expression occurred in the latter.

To determine the SPL function of each genotype, +/+, +/- and -/- flies were homogenized and whole extracts assayed for SPL activity. It was observed that SPL genotype corresponded with SPL activity with +/+ > +/- > -/. Initial evaluation of homozygous mutants indicated that adult SPL mutants were flightless, suggesting a potential defect in either muscle development or energetics of the adult fly. Flight analysis was carried out essentially as described (Vigoreaux, J., J. Saide, K. Valgeirsdottir, and M. Pardue. 1993. Flightin, a novel myofibrillar protein of *Drosophila* stretch-activated muscles. *J Cell Biol.* 121:587-598) by determining the percentage of flies that were flightless or exhibited downward, upward, or lateral flight capabilities in control Canton-S flies as compared to mutant flies.

The transposon was mobilized by crossing SPL mutant flies with flies carrying an actively transcribed transposase gene, which caused the P-element to be excised in the chromosomes of both somatic cells and in the germline. Germline transposon loss is heritable and was identified in progeny by virtue of eye color. Progeny which lost both the transposase

gene and the P-element were then isolated and the restored SPL allele was homozygosed. Progeny which had lost the P-element at the SPL locus demonstrated restoration of flight, indicating that the phenotype correlated with the P-element insertional mutation. To determine the etiology of the flightlessness of  $\text{--/--}$  flies, flies were sectioned through the thoracic region and 5 indirect flight muscles were evaluated by both light and electron microscopy. These studies revealed a reduced number of muscle fibers comprising the dorsal longitudinal muscles with evidence of what appears to be compensatory hypertrophy in the fibers which remained. Electron microscopy revealed no ultrastructural defects in the myocytes which remained.

10 In order to determine whether the loss of SPL expression was due to excess accumulation of S-1-P in the developing adult fly, we salvaged the developing flight muscles of homozygous SPL mutant progeny by adding D,L-*threo*-dihydrosphingosine, an inhibitor of mammalian sphingosine kinase, to the growth media. A significant proportion of homozygous SPL mutant progeny demonstrated restoration of flight when grown on media supplemented with D,L-*threo*-dihydrosphingosine.

15 Northern analysis was performed to investigate SPL expression throughout development. These studies indicated that SPL expression begins at 8-12 hours of embryonic development and remains detectable throughout larval stages and pupation.

20 Therefore, the *Drosophila melanogaster* model described herein can be used to identify pharmacologic suppressors of SPL mutant flies' inability to fly. Drugs which alter SPL activity or expression may be effective treatment for at least some kinds of cancer. Therefore, the fact that a fruitfly SPL null mutant containing a P-element insertion within the SPL coding region is flightless provides an excellent model in which to screen and identify compounds that modulate SPL activity. Thus, other chemicals created through rational drug design approaches can be screened using this method. The *Drosophila melanogaster* model described herein can 25 thus be used to screen an array of rationally designed chemicals with homology to sphingolipids for their ability to restore flight to SPL mutant progeny. Candidate drugs identified using this method can then be further evaluated in an *in vitro* yeast screen.

## Example 8

Further Characterization of Developmental Expression Patterns of SPL in SPL transposon mutant *D. melanogaster*

5 Northern analysis is carried out and extended to include adult samples, and blots are reprobed with SPL specific probes using the following approaches. Once genes are confirmed to encode the predicted enzyme, DNA probes or riboprobes for SPL and S-1-P phosphatase are labeled either radioactively or with digoxigenin. For Northern analysis, full-length probes are labeled by random priming with  $[\alpha-32P]dATP$ . Hybridization is carried out  
10 under standard conditions against an RNA blot prepared from total RNA of flies harvested at different stages of development (embryos at hours 0-4, 4-8, 8-12, 12-24, larval instars 1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup>, early and late pupal stages, and adults). For *in situ* hybridization purposes, <sup>3</sup>H labeling is the most sensitive approach, and the very low energy of the beta particle emitted causes it to travel only short distances through the radiographic emulsion, allowing precise localization for the probe.  
15 However, digoxigenin labeling provides the advantage of being able to visualize hybridization with much higher spatial resolution because of the ability to directly visualize the tissue. Random primer labeling of DNA are performed with either tritium or digoxigenin labeled nucleotides. *In situ* hybridization is performed as described in Blair, S. (Blair S., 2000. Imaginal discs. *In Drosophila Protocols*. W. Sullivan, M. Ashburner, and R. Hawley, editors. Cold Spring  
20 Harbor Laboratory Press, Cold Spring Harbor, NY. 159-175), hereby incorporated by reference in its entirety.

## Example 9

Characterization of sphingolipid species in the *Drosophila melanogaster*

25 Without being bound by theory, it is hypothesized that the phenotype of the SPL mutant *Drosophila* is caused by an abnormal level of S-1-P during development. Further, without being bound by theory, it is the inventors hypothesis that phosphorylated sphingoid base species are responsible for regulating cell proliferation, migration and other events required for

both tumor formation and normal developmental processes in this model organism. Therefore, characterization of sphingolipid species in *Drosophila* was determined.

Method: Wild type (Canton S) whole fly extracts were prepared by mechanical disruption. Lipids were isolated by two-phase extraction and derivatized with the fluorescent molecule o-phthalaldehyde essentially as described in Caligan, et al. hereby incorporated by reference in its entirety (Caligan, T.B., K. Peters, J. Ou, E. Wang, J. Saba, and A.H. Merrill, Jr. 2000. A high-performance liquid chromatographic method to measure sphingosine 1-phosphate and related compounds from sphingosine kinase assays and other biological samples. *Analytical Biochemistry*. 281:36-44). Derivatized lipid extracts were separated by HPLC using a C<sub>18</sub> ODS column (LUNA 4.6 x 250 mm) and mobile phase MeOH/H<sub>2</sub>O/1M TBAP 82:17:0.9, pH 4.8. Standards included commercially available C<sub>10</sub>, C<sub>12</sub>, C<sub>14</sub>, C<sub>16</sub>, C<sub>18</sub> and C<sub>20</sub> sphingosines, as well as the phosphorylated forms of these standards, prepared by incubation of sphingosine standards with extract from a yeast strain which overexpresses the major yeast sphingosine kinase, *LCB4*.

Results: *Drosophila* extracts contained only sphingolipid species which comigrated with C<sub>14</sub> sphingosine and C<sub>14</sub> sphingosine-1-phosphate (S-1-P) standards under the stated conditions. To verify the identity of the peaks in fly extracts which comigrated with C<sub>14</sub>sphingosine and C<sub>14</sub>S-1-P standards, extracts and standards were compared in four different mobile phase buffers. The peak identified as C<sub>14</sub> sphingosine comigrated with the C<sub>14</sub> sphingosine standard under all four conditions (Table 1). However, the peak identified as C<sub>14</sub>S-1-P demonstrated a slight difference from the C<sub>14</sub>S-1-P standard under conditions which exploit differences in charge (MeOH/10 mM KP/1 M TBAP, pH 7.2, 81:18:1).

Table 1: Sphingolipid Identification

	<u>Mobile Phase</u>	<u>C<sub>14</sub>S std</u>	<u>C<sub>14</sub>S in extract</u>	<u>C<sub>14</sub>S-1-P std</u>	<u>C<sub>14</sub>S-1-P in extract</u>
5	MeOH/H <sub>2</sub> O/1M TBAP pH 4.8 82.1:17:0.9	19.1 min	19.0 min	14.8 min	14.8 min
10	MeOH/H <sub>2</sub> O/1M TBAP pH 4.8 79.1:20:0.9	27.3 min	27.1 min	22.5 min	22.1 min
15	MeOH/10mM KP/ 1M TBAP pH 5.5 81:18:1	21.9 min	22.0 min	18.3 min	17.2 min
20	MeOH/10mM KP/ 1M TBAP pH 7.2 81:18:1	21.4 min	21.8 min	15.0 min	17.1 min

This finding is likely to be due to a chemical modification of the phosphate group, since a phosphatase capable of dephosphorylating the C<sub>14</sub>S-1-P standard does not recognize this substrate. Mass spectroscopy is utilized to identify the phosphate group modification of this S-1-P species. Herein, this sphingolipid is referred to as "modified C<sub>14</sub>S-1-P."

#### Example 10

##### Characterization of sphingolipid species in the *Drosophila* SPL mutant

Differences in the quantity or type of sphingolipid species present in mutant versus wild type adult flies and during various stages of development was determined as described below.

Methods were as described in Example 9.

Results: The modified C<sub>14</sub>S-1-P peak was ten-fold higher in the *Drosophila* SPL mutant than in the wild type (using an internal standard to normalize for extraction variation), supporting the notion that the phenotype of the SPL mutant may be due to abnormal accumulation of phosphorylated sphingoid bases and resulting abnormalities in signaling. C<sub>14</sub>

sphingosine was also increased in the mutant, but to a lesser extent (Table 2). No other peaks in the mutant demonstrated a significant difference in comparison to wild type controls.

Table 2: Sphingolipid Quantification (nmol/200 mg flies)

Line (n=3)	modified C <sub>14</sub> S-1-P	C <sub>14</sub> S
5 Canton S (wild type)	0.49 ± 0.07	2.61 ± 0.27
SPL mutant	4.49 ± 0.53	5.27 ± 0.73

10 Example 11

Characterization of the SPL activity encoded by ESTs LP04413/GH3783 and which is absent in insertional mutant 11393

15 *Drosophila* ESTs LP04413 and GH3783 encode a protein with strong homology to other sphingosine phosphate lyases (SPL). Mutant 11393 which demonstrates the flight defect and dorsal longitudinal muscle (DLM) abnormalities described above in Example 7, contains a p-element insertion within this locus. Initial results using a standard SPL assay and a radiolabelled C<sub>18</sub>DHS-1-P substrate indicated that *Drosophila* ESTs LP04413 and GH3783 encode an SPL, 20 since expression restored SPL activity to a yeast SPL mutant. However, the activity conferred by the EST expression in yeast was not pronounced. Since *Drosophila* extracts contain C<sub>14</sub> sphingosine and a modified species of C<sub>14</sub>S-1-P, it was hypothesized that the C<sub>18</sub>DHS-1-P was not a favorable substrate for the major *Drosophila* lyase. Further, residual lyase activity observed in the mutant indicated the presence of more than one SPL activity in *Drosophila*. Therefore, the 25 optimal substrate of the SPL encoded by ESTs LP04413 and GH3783 was determined and this activity was differentiated from other SPL activities in *Drosophila*.

Methods: Wild type (Canton S) and mutant whole fly extracts were prepared by mechanical disruption. Standard SPL assays using C<sub>18</sub> DHS-1-P substrate were performed as previously described (Van Veldhoven, P.P., and G.P. Mannaerts. 1991. Subcellular localization 30 and membrane topology of sphingosine-1-phosphate lyase in rat liver. *J Biol Chem.* 266:12502-12507). An HPLC-based SPL assay was established, to allow for various non-radioactive substrates to be evaluated. For this assay, C<sub>14</sub>S-1-P, C<sub>18</sub>DHS-1-P and modified C<sub>14</sub>S-1-P were

prepared by drying down the lipid extract from 15 mg of 11939 flies, plus 200 pmol C<sub>14</sub>S-1-P standard and 200 pmol C<sub>18</sub>DHS-1-P standard. Lipids were resuspended in 25 µl of 1% Triton X-100 in potassium phosphate buffer, pH 7.4. 175 µl of reaction buffer (KP buffer, NaF, DTT, EDTA, sucrose) were added, and mixture was tip sonicated for 20 seconds, followed by addition 5 of 50 µg of protein from whole cell extract of flies (CS or 11939) or Δdpl1:lcb4 yeast overexpressing the fly lyase. Incubation proceeded for 1 hr at 37°C. Reaction was stopped by adding 175 µl of MeOH containing 0.2% acetic acid. The reaction was applied to STRATA C18 column in 40% MeOH containing 0.1% acetic acid. The column was washed with 600 µl of 40% MeOH containing 0.1% acetic acid. Lipids were eluted with 1ml of 90% MeOH/10% 10 mM K-  
10 Phosphate, pH 7.2. Samples were dried and resuspended in MeOH, treated with o-phthalaldehyde and injected on the HPLC. The degradation of S-1-P standards and modified C<sub>14</sub>S-1-P were compared to standards incubated in the absence of protein extracts.

15 **Results:** An activity which metabolizes modified C<sub>14</sub>S-1-P is present in wild type fly extracts but is absent in the mutant fly extracts. Residual SPL activity does exist in the mutant fly. This activity is distinct from that encoded by LP04413/GH3783, in that it metabolizes C<sub>14</sub>S-1-P and C<sub>18</sub>DHS-1-P with an efficacy similar to or better than wild type. The pH curve of the residual SPL activity in mutant flies is identical to that seen in wild type flies (against a C<sub>18</sub>DHS-1-P substrate), indicating that this activity is not disrupted in the mutant.

20

Example 12  
Further characterization of the *Drosophila melanogaster* SPL mutant phenotype

25 Adult SPL mutant flies demonstrated inability to fly and abnormal patterning of indirect flight muscles. The adult SPL mutant flies consistently demonstrated abnormal patterning of DLMs, although the number of remaining DLMs varied in each mutant. In this Example, it was determined whether the abnormal muscle development was limited to the adult fly, or whether the defect was also present at earlier developmental stages.

**Methods:** Larval locomotor assay. Third instar larvae were placed on a clear agar substrate that overlays a grid. A light source at one end provided a phototactic stimulus. Distance traveled was scored during three minute trials. Larval muscle microscopy. Larvae were filleted during the third instar and pinned with the dorsal cuticle down. The viscera were removed to 5 allow an unobstructed view of the body wall muscles using polarized light. Muscles were refractile due to the presence of filamentous arrays in each muscle fiber.

**Results:** 11393 mutant larvae demonstrated significant defects in locomotion in comparison to wild type larvae, although phototactic response is intact. In all mutant larvae examined, the T2-dorsal oblique muscles exhibited alterations in number and/or size. Fused, hypertrophied residual dorsal obliques were observed in the mutants.

Since the four pairs of dorsal obliques in thoracic segment two create scaffolds which give rise during pupation to the DLM structures of the adult, it is likely that the developmental defect seen in the adult is the result of a process which begins much earlier, during larval development or embryogenesis.

10  
15  
20  
25  
30  
35  
40  
45  
50  
55  
60  
65  
70  
75  
80  
85  
90  
95  
100

### Example 13 Human SPL Expression Patterns in Cancer

20 To determine if SPL expression is altered in human tumors, we utilized a cancer profiling array which contains more than 240 cDNA pairs representing tumor tissue and corresponding normal tissue from the same patient. By utilizing tissue pairs from one patient, differences between gene expression in tumor and normal tissue which might be due to person to person variability should not confound the interpretation of results. Additionally, each blot was 25 normalized for loading using four separate housekeeping genes. Traditional hybridization techniques were utilized to probe this cDNA blot with a 300 nucleotide 3' fragment of human SPL cDNA (SEQ ID NO:7), which was obtained from the previously described cloning experiments. Analysis of the array indicated that, whereas human SPL expression is matched closely in most tissue pairs, it is significantly reduced in colon cancer specimens, with a 50%

reduction in expression in colloid cancer of the colon and 61% reduction in adenocarcinoma of the colon. Reduced SPL expression was also seen in adenocarcinoma of the uterus. None of the tumors in which SPL expression is diminished demonstrate SK overexpression. Thus, altered SPL expression is observed in primary human tumors. Therefore, modulating the activity of SPL 5 protein either by altering gene expression or through direct action on the protein may provide a useful treatment for individuals afflicted with an SPL-related cancer. Furthermore, SPL expression serves as a useful diagnostic marker of cancer in humans.

10 From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for the purpose of illustration, various modifications may be made without deviating from the spirit and scope of the invention.